THE RESPONSE OF *SACCHAROMYCES CEREVISIAE* TO FERMENTATION UNDER CARBON DIOXIDE PRESSURE

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When laboratory fermenters containing 4 litres of wort were maintained under a variety of conditions at an excess pressure of 2 atm of carbon dioxide throughout fermentation by *Saccharomyces cerevisiae* (NCYC-1108), the fermentation rate, yeast growth and final concentration of fusel oils all decreased and the final pH increased. This agrees with reported work on the effect of carbon dioxide pressure on fermentation by *S. carlsbergensis*. The effect of carbon dioxide pressure on the production and removal of vicinal diketones and their precursors by *S. carlsbergensis* is known to be variable but, except at very low temperatures, pressure has either no effect or increases the rate of removal of these compounds in the final stages of fermentation. In the present experiments with a strain of *C. cerevisiae*, however, carbon dioxide pressure always led to the fresh beer having a higher lever of vicinal diketones and their precursors than the control fermentation, even at temperatures up to 20°C.

Key words: *carbon dioxide, pressure fermentation, S. cerevisiae, fusel oils, vicinal diketones, diacetyl, maturation.*

Introduction

Carbon dioxide pressure has been used as a control device during fermentation and maturation in many breweries since its proposal in the early 1960s.[1,4,5,12] The biochemical mechanisms of carbon dioxide action are still not clear but seem to be relatively complex. At low concentrations, equivalent to pressures up to about 0·2 atm, carbon dioxide tends to stimulate yeast growth.[9] This is thought to be because of its use as a substrate in carboxylation reactions and, in support of this idea, the provision of a rich medium, especially one that contains aspartic acid, significantly reduces both the stimulatory effect of carbon dioxide and the amount of carbon incorporated from this source.[9] Once pressures of 0·3 to 0·5 atm are reached, carbon dioxide begins to have an inhibitory effect.[3] The tricarboxylic acid cycle seems to be particularly sensitive so that respiration is severely reduced by relatively low levels of carbon dioxide.[8] In contrast, alcohol production is still unaffected at 4 atm.[3] At about 2·5 to 3·0 atm carbon dioxide is reported to prevent cell division completely[8] and so systems such as brewery fermentations which depend on a certain amount of cell growth are unlikely to be able to function above about 2 atm depending on the exact fermentation temperature. Within this zone of 0–2 atm there should be little effect of carbon dioxide on fermentation rate per cell although in practice a reduction in growth rate would be expected at the higher end of the pressure range and this would, of course, extend the period needed to achieve a given level of attenuation.

Brewing practice has usually been devised empirically using a combination of higher temperature to speed the process and carbon dioxide pressure to reduce the undesirable effect of the higher temperature.[5] The pressure is commonly applied towards the end of fermentation to gain the maximum benefits with the minimum disadvantage. A number of workers have reported on the effects of carbon dioxide pressure on intermediate scale fermentations. All agree that the fermentation rate can be lowered and the fusel oil content of the final beer reduced. However, production and elimination of the vicinal diketones and their precursors has shown different patterns in different systems. When Meindaner *et al*[7] applied pressure toward the end of fermentation and maintained this throughout maturation they saw little effect on the rate of removal of these compounds. In contrast, Kumada *et al*[6] found a pronounced drop in vicinal diketone precursor levels during pressure fermentation at 14·5°C and 18°C although at 9°C the pressure seemed to cause distinctly increased levels. Rice *et al*[11] showed that pressure decreased vicinal diketone concentrations but because of the higher temperatures involved the final beer could still have a higher diacetyl level than the control produced at a lower temperature.

All reports in the brewing literature dealing with carbon dioxide pressure seem to refer to lager production with *Saccharomyces carlsbergensis*. Becasue of this, and the variation in response of vicinal diketone production to carbon dioxide, the present work was carried out to confirm the effect of carbon dioxide on a brewing strain of *S. cerevisiae* and, in particular, to discover the effect on the production and removal of vicinal diketone precursors.

Materials and methods

Yeast storage and propagation.—The yeast strain used in all the experiments was *Saccharomyces cerevisiae* NCYC 1108. The cultures were stored on Sabouraud agar slopes at 5°C. When required for experimental purposes, yeast was inoculated into 300 ml Sabouraud broth in a 500 ml conical flask and incubated for 3 days at 20°C. The yeast crop was separated by centrifugation and washed twice in distilled water before use.

Wort preparation.—800 g of wort powder, obtained from Distillers Co Ltd were dissolved in 8·5 litres of distilled water. The wort was boiled for 1 h, filtered and autoclaved at 10 psi for 10 min. The final pH was 5·4–5·5 and the specific gravity about 1·037.

Fermentation.—Sterilised wort was inoculated with washed yeast paste, collected by centrifugation, at a rate of 3 g/litre to give an initial cell count of $8–10 \times 10^6$ per ml and 4 litre aliquots transferred to each of two 11·41 Cornelius 'Spartan' steel tanks. In the case of one tank (the control) the safety valve was replaced by a fermentation lock and in the other tank (the experimental fermentation) the safety valve was replaced by a release valve set to open at an excess pressure of 28 psi (2 atm). Immediately after addition of the wort the experimental fermenter was brought up to pressure using a cylinder of carbon dioxide. Temperature control was achieved by attemporating the wort to the desired temperature before inoculation and then carrying out the fermentation in an Astell temperature-controlled cabinet.

Samples for analysis were withdrawn as required from the outlet in the side of the fermenter and the pressure was restored immediately after sampling by introducing carbon dioxide from a cylinder into the headspace of the fermenter.

Analyses.—The concentration of n-propanol, iso-butanol and mixed iso-amyl alcohols was determined by injection of 1 µl of clarified medium into a Perkin-Elmer F11 GC

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TABLE I. Effect of Gas Pressure on Fermentation Characteristics

Temperature °C	Pressurising agent	Fermentation speed*	Final fusel oil concentration (ppm)			Final cell number × 10^7	Final pH
			n-propanol	iso-butanol	iso-amyl alcohols		
20	CO_2	37	6·0	31·0	69·0	7·1	4·65
	–	18	14·5	52·5	86·0	9·2	4·25
~16	CO_2	54	6·0	33·5	65·0	6·6	4·75
	–	36	13·5	41·5	74·0	8·5	4·25
16†	CO_2	44	8·5	56·0	75·0	6·6	4·35
	–	32	14·5	68·0	95·0	8·9	3·95
12	CO_2	116	ND	18·5	33·0	6·4	4·85
	–	58	5·0	43·5	68·5	7·5	4·55

*hours required to reach half the maximum ethanol concentration attained in the control.
†agitated (see Results section).
ND–not detectable.
The results shown are the averages of two fermentations carried out on separate occasions.

fitted with a 2 m glass column, ⅛ in i.d., packed with 20% Carbowax 1500 on chromosorb W 100–200 mesh with 20% Carbowax 20 M in the injection zone. Operating conditions were: oven temperature, 80°C; injection zone temperature, 250°C; FID detector temperature, 146°C; nitrogen carrier inlet pressure, 20 psi; hydrogen inlet pressure 18 psi and air inlet pressure, 20 psi.

Ethanol was measured using the same GC system but with an oven temperature of 75°C.

The concentration of vicinal diketone precursors was measured using the Institute of Brewing Recommended Method with the substitution of a conventional distillation flask for the Markham still.

Yeast cell numbers were estimated microscopically using a Neubauer haemocytometer.

Results and discussion

In all the pressurised fermentations reported in this paper the gas pressure was applied immediately after inoculation of the yeast into the wort and then maintained throughout the experimental period in order to maximise any effects. The results shown in Table I indicate that at all the temperatures tested an excess carbon dioxide pressure of 28 psi (2 atm) applied to unagitated fermentations significantly slowed ethanol production, reduced the final concentration of fusel oils, reduced yeast growth and increased final pH.

The most simple explanation of these results is that the slower fermentation, lower fusel oil concentration and higher beer pH are consequences of the reduced yeast growth (Table I) and the generally lower level of cells in suspension (Fig. 1.). In order to examine this idea, an experiment was carried out where the yeast in both control and pressurised fermenters was resuspended three times each day by gently swirling the fermenters. The results in Table I show that this treatment caused a slight increase in the speed of fermentation of the control but a distinct increase in the pressurised vessel: reductions of 4 h and 10 h respectively for the time taken to reach half the maximum ethanol concentration achieved in the control. However, the total yeast crop was unaffected by the resuspension treatment so it seems that the faster fermentation under this condition must be due to improved mixing of yeast and fermentation medium. These results are in line with published work which indicates that cell division is prevented by pressures above 2·5 atm[8] whereas fermentative capacity is not reduced until much higher pressures are reached.[3,10] In the swirled fermenters final pH levels were lower and fusel oil concentrations higher than in the stationary fermenters under otherwise the same conditions but the relative effect of carbon dioxide seemed to remain the same, however. These results suggest that the influence of carbon dioxide during fermentation is not simply due to a single inhibitory effect on yeast growth which causes proportional changes in resultant properties but rather its action is multivalent and so it can be expected that different areas of yeast metabolism will be affected to different extents. Such a view is supported by reports that after a number of generations of growth under carbon dioxide pressure some yeast strains begin to lose their viability[12] and, as mentioned above, it has been established that respiration is much more sensitive to carbon dioxide than is fermentation. Further support for this idea is contained in Table I which shows that under all the conditions tested the production of fusel oils was not uniformly inhibited by carbon dioxide but n-propanol concentration was the most sensitive followed by iso-butanol with the iso-amyl alcohols always being the least affected.

When the precursors of vicinal diketones were monitored throughout the fermentations a relatively consistent pattern emerged (Fig. 1). Carbon dioxide pressure tended to increase the maximum concentration reached and the time which it was attained and, after the maximum point the concentration of vicinal diketones precursors usually declined more slowly in the pressurised fermenter than in the control.

Exactly what happened depended on the temperature of fermentation but the result in all cases was that the freshly fermented beer produced under pressure had a distinctly higher concentration of vicinal diketones and their precursors than the unpressurised control. At 20°C, (Fig. 1a) both experimental and control fermentations reached their maximum vicinal diketone precursor level at very similar times but the concentration in the pressurised vessel was *ca* 40% greater than in the control. Subsequent removal of the vicinal diketones and their precursors was faster in the control so that after 140 h of fermentation the control contained *ca* 0·1 mg litre^{-1} of 'diacetyl' whilst the pressure fermentation still contained *ca* 0·6 mg litre^{-1}. The number of yeast cells in suspension was always greater in the control and this could directly explain the slower absorption of the vicinal diketones in the later part of the fermentation. However, how a lower cell number could produce a higher maximum concentration of vicinal diketone precursors in the first phase of fermentation without some effect of carbon dioxide on the chemistry of the process is not clear. At 16°C, (Fig. 1b) the experimental fermenter again reached a maximum

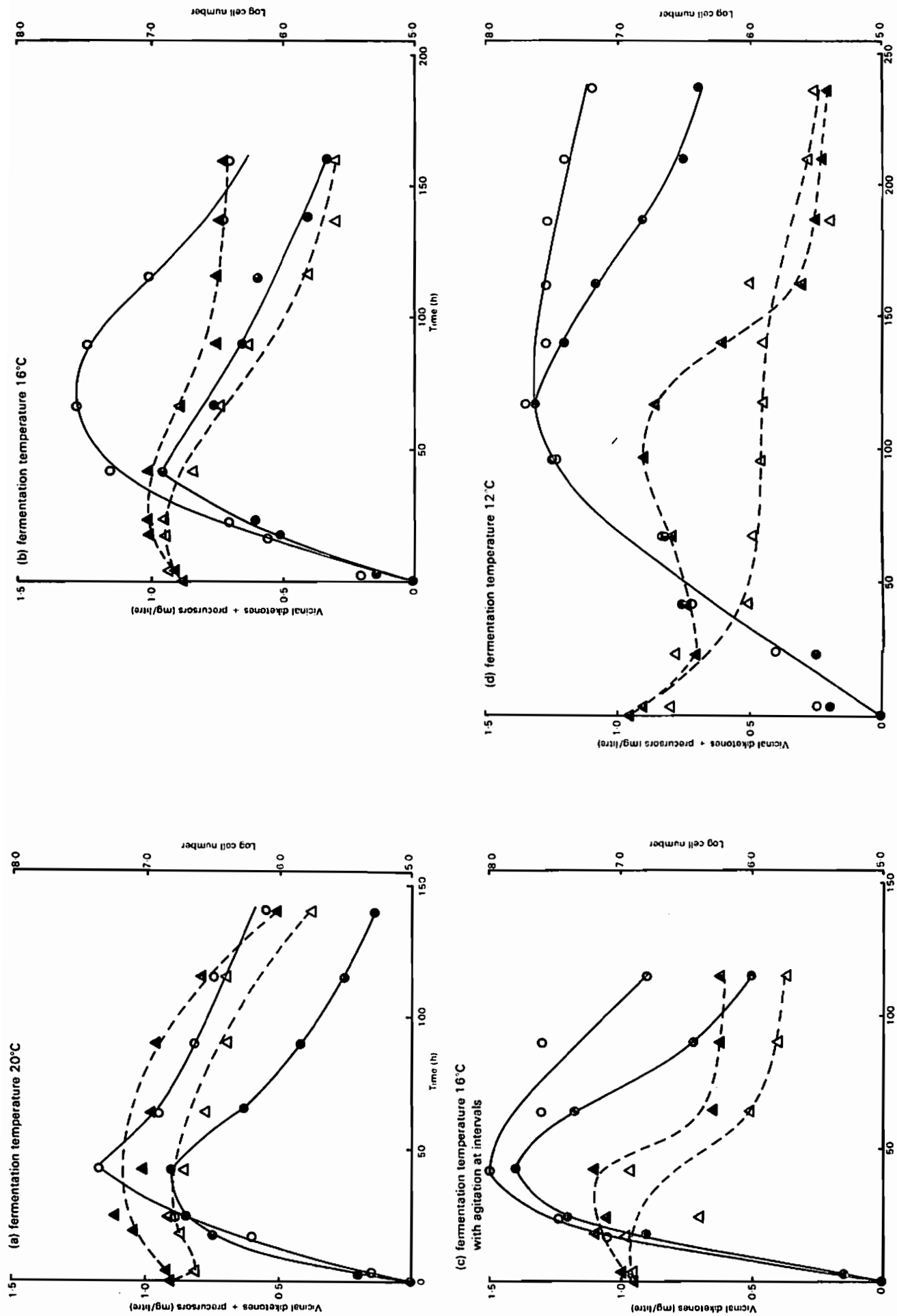

Fig. 1. Effect of carbon dioxide pressure on yeast cell numbers in suspension and on the production and removal of the vicinal diketones and their precursors. Experimental conditions are given in the text. The results shown are the averages of two fermentations carried out on different occasions and refer to the experiments whose other results are given in Table 1. Vicinal diketones and their precursors: ●, control; ○, under pressure. Cell numbers in suspension: ▲, control; △, under pressure.

concentration of vicinal diketone precursors *ca* 40% greater than the control but took distinctly longer to reach this point. The rate of disappearance of the vicinal diketone precursors in the experimental fermentation was then very comparable to, and certainly not slower than that in the control when considered at the same substrate concentration despite there being a much greater number of cells in suspension in the control fermenter at the equivalent times. The supposition that more is involved than just a cell number effect is confirmed by the experiment at 12°C (Fig. 1d) where, under rather extreme conditions for *S. cerevisiae*, there is clearly no correlation between cell number and the production or removal of vicinal diketone precursors and by the experiment at 16°C where the fermenter was agitated three times a day (Fig. 1c). This treatment, although it altered the speed of fermentation (Table I), the shape and maximum concentration of the vicinal diketone precursor profile, still resulted in more or less the same concentration of vicinal diketone precursors in both fermenters after 120 h as occurred in the unagitated fermentation.

There are many possible points at which carbon dioxide could act to produce the patterns shown in Fig. 1. The effect could be at the cell membrane affecting the leakage of the α-acetohydroxy acids and the uptake of the resultant diketones. It has already been demonstrated that carbon dioxide can affect the fatty acid composition of yeast cell membranes.[2] Alternatively, the point of action could be individual metabolic reactions within the cell and it is also possible that dissolved carbon dioxide could reduce the rate of the spontaneous decomposition of the α-acetohydroxy acids in the fermenting wort so preventing formation of diacetyl and 2,3-pentanedione which are the compounds taken up by the yeast. The last hypothesis seems the most unlikely as if it were true, then it might be expected that carbon dioxide pressure would always delay the removal of the vicinal diketone precursors and this is known not to be the case. Given the variability in response to carbon dioxide which has been reported in this area a direct effect on the yeast is more likely. Whatever the mode of action, it is, however, quite clear from Fig. 1 that carbon dioxide pressure fermentation with *S. cerevisiae* may result in increased levels of vicinal diketones in contrast to most of the reported experiences with *S. carlsbergensis*.

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